Histamine H₃ receptors modulate the release of [³H]-acetylcholine from slices of rat entorhinal cortex: evidence for the possible existence of H₃ receptor subtypes

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1 The effect of agents which interact with the histamine H₃ receptor on potassium-stimulated tritium release from slices of rat entorhinal cortex preloaded with [³H]-choline is described. We have examined the effects of the selective H₃ receptor agonist, (R)- α -methylhistamine (RAMH), and a number of H₃ receptor antagonists, including the selective compound thioperamide, on the potassium-stimulated release of tritium.

2 In the presence of mepyramine and ranitidine, RAMH (0.01–10 μ M) inhibited potassium-stimulated tritium release in a concentration-dependent manner, EC₅₀ = 0.11 μ M. The maximum inhibition was approximately 50%.

3 Thioperamide displaced the RAMH concentration-response curve to the right yielding a pK_B value of 8.4. There was no change in the maximum response to RAMH.

4 Other H₃ receptor antagonists, including impromidine and burimamide, also caused rightwards displacement of the linear portion of the RAMH concentration-response curve. However, phenylbutanoylhistamine and betahistine, which are reported to be relatively potent H₃ receptor antagonists, showed very low affinity.

5 Thioperamide (0.001–1 μ M) alone enhanced the potassium-stimulated release of tritium in a concentration-dependent manner. Maximum effects were observed at 0.1–1 μ M thioperamide, enhancing release by approximately 20%.

6 Results are discussed in terms of the regulatory role of H₃ receptors on acetylcholine release and the possible existence of H₃ receptor subtypes.

Keywords: Acetylcholine release; entorhinal cortex; histamine H₃ receptors

Introduction

The existence of a third histamine receptor pharmacologically distinct from both the H₁ and H₂ receptor was suggested by Arrang and colleagues in 1983. This histamine receptor, termed H₃, was originally characterized as a presynaptic autoreceptor modulating histamine synthesis and release in slices of rat cerebral cortex. With the more recent discovery of a selective agonist, (R)- α -methylhistamine (RAMH), and a selective antagonist, thioperamide, for this receptor (Arrang *et al.*, 1987), other workers have reported that H₃ receptors also function as presynaptic inhibitory heteroreceptors controlling the release of noradrenaline (Schlicker *et al.*, 1989) and 5-hydroxytryptamine (Schlicker *et al.*, 1988) in the central nervous system. These receptors also appear to modulate the release of non-adrenergic non-cholinergic transmitters (Ichinose & Barnes, 1989; Taylor & Kilpatrick, 1992) and cholinergic transmission (Trzeciakowski, 1987; Ichinose *et al.*, 1989) in the periphery. Indeed, it has recently been demonstrated that activation of the H₃ receptor inhibits the release of tritium, presumably [³H]-acetylcholine, from the guinea-pig isolated longitudinal muscle myenteric plexus preincubated with [³H]-choline (Poli *et al.*, 1991).

We now describe the development and preliminary characterization of an *in vitro* model to investigate H₃ receptor-mediated modulation of acetylcholine release in the central nervous system. Parts of this study have been presented, in abstract form, to the British Pharmacological Society (Clapham & Kilpatrick, 1992).

Methods

Preparation of slices of rat entorhinal cortex

Male Lister Hooded rats (250–300 g, Glaxo bred) were stunned and decapitated. The brains were removed and cooled on ice. The entorhinal cortex was dissected from each brain (10 rats per experiment, each providing two entorhinal cortices) and placed on dry filter paper. The tissue was chopped transversely through the cortical layers with a McIlwain chopper to produce 0.3 mm slices. The pooled cut tissue was placed in a 25 ml sterilin tube containing oxygenated (95% O₂/5% CO₂) Krebs solution. The slices were then separated by shaking and washed (3 $\times$ 20 ml) in Krebs solution. The Krebs solution was replaced by Krebs solution containing 39.75 mM KCl (NaCl was decreased to 83 mM to maintain tonicity) at 37°C for 20 min. This depolarization procedure was included to deplete endogenous neurotransmitters and enhance the subsequent uptake of [³H]-choline.

After washing thoroughly with fresh Krebs solution (4 $\times$ 20 ml) the slices were incubated at 37°C for 40 min in 5 ml Krebs containing [³H]-choline chloride (0.1 μ M; 87 Ci mmol⁻¹, New England Nuclear). The slices were then washed with fresh Krebs solution (4 $\times$ 20 ml) in order to remove excess radioactivity. Approximately 100 μ l of settled slices were placed in each of 20 superfusion chambers which were then superfused with Krebs solution at 37°C (flow rate 0.5 ml min⁻¹) containing 1 μ M hemicholinium for 60 min.

Experimental design

After a 60 min equilibration period, samples of superfusate were collected every 4 min. Two stimulation methods were examined. Samples were either depolarized at 12 min (S₁) and

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48 min (S_2) and 20 samples per chamber collected; or slices were depolarized once only at 12 min (S_1) and 10 samples were collected. Depolarization was achieved by changing the superfusing solution to Krebs buffer containing 20 mM KCl for 4 min (tonicity was maintained by reducing the NaCl concentration to 103 mM).

When the double stimulation method was used, drugs were included in the perfusate 20 min before the S_2 stimulation to the end of the experiment. Under these conditions the potassium-evoked tritium release was calcium-dependent (results not shown).

With the single stimulation method, antagonists were present in the perfusate from the start of the 60 min equilibration period onwards and agonists were included 20 min before the S_1 stimulation to the end of the experiment. Antagonist effects were quantified on their ability either to displace the full RAMH concentration-response curve (CRC) to the right or to shift a 2-point RAMH CRC (0.1 and 1 μ M) in a similar fashion.

Quantification of tritium released

Acetylcholine release was estimated from the outflow of tritium from the tissue. This was measured by liquid scintillation spectrometry (5 min count time/sample), 10 ml of Emulsifier Safe scintillation fluid (Packard) being added to each 4 min sample of superfusate. At the end of the experiment the tissue slices from each chamber were solubilized in 1 ml of Soluene-100 (Packard) and the tritium was determined. The disintegrations per min for each 4 min collection period were converted to fractional release by dividing by the total amount of radioactivity present in the tissue at the start of that 4 min collection period. The stimulated release was calculated as the difference between potassium-evoked and basal release.

Data analysis

The results are expressed as either the mean S_2/S_1 ratio or the mean fraction released by $S_1 \pm$ standard error of the mean of n determinations. These data are then expressed as a percentage inhibition or stimulation of the Krebs control response. Concentration-response curve data were analysed by the programme 'ALLFIT' to yield pD_2 values. Antagonist pK_B values were estimated from the equation:

$$pK_B = \log(\text{concentration ratio} - 1) - \log(\text{concentration of antagonist})$$

Statistical analyses were performed by use of the unpaired t test.

Drugs and solutions

The composition of Krebs-Henseleit medium used (mM): NaCl 118, NaHCO_3 25, KCl 4.75, KH_2PO_4 1.2, CaCl_2 1.25, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2 and glucose 11.

Drugs were dissolved in water and diluted in Krebs-Henseleit medium.

Gifts of burimamide and impromidine trihydrochloride (SmithKline Beecham) and mepyramine maleate (Rhône Poulenc) are gratefully acknowledged. Betahistine dihydrochloride was purchased from Aldrich Chemical Company Ltd. (U.K.) and hemicholinium-3 from Sigma Chemical Company Ltd. (U.K.). Thioperamide, (*R*)- α -methylhistamine hydrochloride, ranitidine hydrochloride and phenylbutanoylhistamine were synthesized by the Chemistry Research Department, Glaxo Group Research.

Results

Potassium-evoked release of tritium from slices of rat entorhinal cortex preloaded with [^3H]-choline

The inclusion of potassium chloride (K^+ ; 20 mM) in the perfusate (S_1) for 4 min resulted in a release of tritium

Figure 1 Data from a typical experiment using slices of rat entorhinal cortex showing the fractional release of tritium induced by potassium (20 mM) applied at S_1 for 4 min.

(Figure 1). The fraction released by S_1 stimulation was routinely between 0.05 and 0.07. A second exposure to potassium (S_2) resulted in a similar release of tritium. Using this 'double stimulation method' control S_2/S_1 ratios were routinely between 0.8 and 1.2 (Figure 2).

Effect of (*R*)- α -methylhistamine (RAMH) assessed using two different stimulation methods

Addition of RAMH (0.3, 1 and 3 μ M) to the perfusate 20 min before S_2 resulted in a concentration-dependent inhibition of tritium release from the rat entorhinal cortex on the second stimulation (Table 1). The highest concentration of RAMH tested (3 μ M) induced an inhibition of approximately 30%.

Figure 2 Data from a typical experiment using slices of rat entorhinal cortex showing the fractional release of tritium induced by potassium (20 mM) applied at S_1 and S_2 for 4 min.

Table 1 Effect of (R)- α -methylhistamine (RAMH) on tritium release from slices of rat entorhinal cortex by the double potassium stimulation method

Treatment (μ M)	Evoked tritium release S ₂ /S ₁ ratio	% Inhibition of control (n)
K ⁺ control	0.92 $\pm$ 0.02	(11)
RAMH (0.3)	0.79 $\pm$ 0.03*	(6) 14.1
RAMH (1.0)	0.70 $\pm$ 0.03*	(15) 23.9
RAMH (3.0)	0.62 $\pm$ 0.01*	(5) 32.6

Values shown are the mean $\pm$ s.e.mean of data taken from 4 experiments (n = number of superfusion chambers per treatment) *P < 0.005 vs control

In a single experiment, addition of RAMH (3 μ M) to the perfusate 20 min before S₁ resulted in an inhibition of approximately 40% (results not shown) i.e. greater than was obtained with the S₂/S₁ system. When the two stimulation methods were compared directly in the same experiment, RAMH (3 μ M) inhibited K⁺-evoked tritium release from the entorhinal cortex by approximately 22% by the S₂/S₁ method and by 44% by the S₁ only method (Figure 3). In subsequent experiments, therefore, the single stimulation method was employed.

Effect of mepyramine and ranitidine on RAMH-induced inhibition of tritium release

Inclusion of mepyramine (3 μ M) and ranitidine (10 μ M) in the perfusing Krebs solution had no significant effect on either basal tritium release or K⁺-evoked tritium release. In the absence of mepyramine and ranitidine the mean control (K⁺) fraction released was 0.067 $\pm$ 0.005 (n = 4 experiments). When the H₁ and H₂ antagonists were included the fraction released was 0.071 $\pm$ 0.004 (n = 5 experiments). The apparent pD₂ value of RAMH in the absence of mepyramine and ranitidine was 5.9 (Hill slope = 0.64); this was increased to 7.1 (Hill slope = 0.88) when mepyramine and ranitidine were included (Figure 4). There did not appear to be any change in the maximum inhibitory response to RAMH. The effect of the inclusion of either mepyramine (3 μ M), ranitidine (10 μ M)

Figure 3 The effect of (R)- α -methylhistamine (RAMH) on potassium-evoked tritium release using two different stimulation methods. Values shown are the mean $\pm$ s.e.mean (vertical bars) of data taken from 3–4 superfusion chambers per treatment.

Figure 4 The effects of the H₁ receptor antagonist, mepyramine and the H₂ receptor antagonist, ranitidine on (R)- α -methylhistamine (RAMH)-induced inhibition of tritium release. RAMH (●) pD₂ = 5.9, RAMH in the presence of mepyramine (3 μ M) and ranitidine (10 μ M) (○), pD₂ = 7.1. Values shown are the mean $\pm$ s.e.mean (vertical bars) of data taken from 4 experiments, 3–4 superfusion chambers per treatment.

or a combination of the two antagonists was examined. At 0.3 μ M RAMH alone induced an inhibition of 10.8%. This effect was increased to 17.6% in the presence of mepyramine and to 21.6% in the presence of ranitidine. However, when both antagonists were present, the greatest inhibitory effect of RAMH (0.3 μ M) was observed, 29.7%. Both antagonists were therefore included in subsequent experiments.

Effect of thioperamide on RAMH-induced inhibition of tritium release

Thioperamide (0.03 μ M) displaced the RAMH concentration-response curve (CRC) to the right in a parallel fashion with no decrease in the maximum inhibition. A pK_B value of 8.4 was calculated (Figure 5; RAMH Hill slope = 0.43, RAMH in the presence of thioperamide Hill slope = 0.35). Thioperamide (0.03 μ M) alone appeared to enhance tritium release in 3 out of the 5 experiments; control fraction released = 0.052 $\pm$ 0.003 (n = 10 chambers), thioperamide fraction released = 0.062 $\pm$ 0.009 (n = 8 chambers). However this effect did not reach the level of statistical significance.

Effect of thioperamide on potassium-evoked tritium release

Inclusion of thioperamide (1 μ M) in the perfusate 60 min before S₁, induced an increase in K⁺-evoked tritium release of 23.0 $\pm$ 6.7% (n = 3 experiments). When a concentration-response curve was constructed for thioperamide (0.001–1 μ M) in a separate series of experiments, maximum stimulatory effects of approximately 20% were observed between 0.1 and 1 μ M (Figure 6). The half maximal effect of thioperamide was achieved at 6.0 $\pm$ 2.9 nM (pD₂ = 8.2).

Determination of the effects of H₃ receptor antagonists using a 2-point RAMH CRC

Five H₃ receptor antagonists were tested at a single concentration expected to displace a linear portion of the RAMH CRC approximately 10 fold. Apparent parallel rightwards displacements of the RAMH CRC were obtained with thioperamide, burimamide, impromidine and betahistidine (compounds listed in order of potency); however phenylbutanoylhistamine had no effect. Concentrations tested together with calculated pK_B values are presented in Table 2.

Figure 5 The effect of the H_3 receptor antagonist, thioperamide on (R)- α -methylhistamine (RAMH)-induced inhibition of tritium release. RAMH ($\bullet$), RAMH in the presence of thioperamide ($0.03 \mu\text{M}$) ($\circ$), thioperamide $pK_B = 8.4$. Values shown are the mean $\pm$ s.e.mean (vertical bars) of data taken from 5 experiments, 3–4 superfusion chambers per treatment.

Figure 6 The effect of the H_3 receptor antagonist, thioperamide on potassium (20 mM)-evoked tritium release. Values shown are the mean $\pm$ s.e.mean (vertical bars) of data taken from 3–6 experiments.

Table 2 Apparent affinity values of H_3 receptor antagonists for antagonism of (R)- α -methylhistamine (RAMH)-induced inhibition of tritium release

Antagonist	Concentration tested (μM)	Approximate pK_B value	Number of experiments
Thioperamide	0.03	9.0 ± 0.1	3
Impromidine	3	6.3 ± 0.2	3
Burimamide	3	7.1 ± 0.4	3
Phenylbutanoyl-histamine	10	< 5	2
Betahistidine	100	4.5 ± 0.3	3

Values shown are the mean $\pm$ s.e.mean

Discussion

In this paper we describe a preliminary characterization of the effects of the selective H_3 receptor agonist, RAMH, and a number of H_3 receptor antagonists including the selective compound, thioperamide, on the calcium-dependent, potassium-stimulated release of tritium from slices of rat entorhinal cortex preloaded with [^3H]-choline. Barnes *et al.* (1989) have previously demonstrated that, using a similar methodology but including an acetylcholinesterase inhibitor in the perfusing Krebs medium, the basal tritium release is predominantly

[^3H]-choline, but the release stimulated by potassium is [^3H]-acetylcholine and the levels of [^3H]-choline are too small to be defined.

Initial observations revealed that, when using the double potassium stimulation method and calculating an S_2/S_1 ratio, RAMH ($3 \mu\text{M}$) inhibited tritium release by some 30%. However, the largest effects of RAMH, inhibiting release by approximately 50% at 3–10 μM , were observed when a single potassium stimulation was applied to the tissue slices. We have no good explanation for this at present. The slope of the RAMH inhibition curve appeared to be shallow. This may have been due to experimental variability. Alternative explanations include the involvement of more than one receptor and further studies are clearly required.

The maximal inhibitory effect of RAMH is similar to that observed in the histamine release assay (Arrang *et al.*, 1987) in which RAMH is reported to inhibit potassium-evoked [^3H]-histamine release from slices of rat cerebral cortex pre-loaded with [^3H]-histidine by up to 60%. However, in the noradrenaline (Schlicker *et al.*, 1989) and 5-hydroxytryptamine (Schlicker *et al.*, 1988) release assays, the inhibitory effects of RAMH are considerably less, maximum inhibition being approximately 25%.

RAMH is a selective H_3 receptor agonist, but it is likely that H_1 and H_2 receptors are involved in its regulation of [^3H]-acetylcholine release. Thus, when experiments were performed in the presence of mepyramine and ranitidine, the potency of RAMH increased approximately 10 fold. The control potassium stimulated release of tritium, however, was unaffected by the inclusion of these antagonists. Preliminary studies in which ranitidine and mepyramine were studied separately (results not shown) indicate that both H_1 and H_2 receptors were involved in the response to RAMH and the effects of H_1 and H_2 receptor blockade were additive. The precise mechanisms involved are unknown, but one can postulate that H_1 and H_2 receptor stimulation may enhance acetylcholine release. In histamine release assays (Arrang *et al.*, 1983) the inhibitory action of exogenous histamine is unaffected by the presence of mepyramine and cimetidine. In addition, the pD_2 value for RAMH is 8.4 (Arrang *et al.*, 1987), over 10 fold greater than that observed in the acetylcholine release assay. The difference may be explained by a lower receptor reserve or less efficient coupling in the [^3H]-acetylcholine release assay.

Confirmation that the inhibitory effects of RAMH on acetylcholine release are mediated through the H_3 receptor come from antagonist studies. Thioperamide antagonized the effect of RAMH yielding a pK_B value of 8.4, or 9.0 as calculated with a 2 point curve. These values are in agreement with its antagonist potency in other *in vitro* preparations of the H_3 receptor (e.g. $pK_i = 9.0$, Arrang *et al.*, 1987; $pK_B = 9.0$, Hew *et al.*, 1990; $pK_B = 8.1$, Taylor & Kilpatrick, 1992). Similarly, burimamide ($pK_B = 7.1$) and impromidine ($pK_B = 6.3$) antagonized the effects of RAMH with potency values close to those reported in other *in vitro* assays of the H_3 receptor ($pK_i = 7.2$ and 7.2 respectively, Arrang *et al.*, 1983; $pK_B = 6.8$ and 6.6 respectively, Taylor & Kilpatrick, 1992). However, phenylbutanoylhistamine (PBH; 10^{-5} M)

Table 3 Apparent affinity values of phenylbutanoyl-histamine (PBH) and betahistidine in four *in vitro* models of the H_3 receptor

H_3 antagonist	[^3H]-ACh release pK_B	[^3H]-HA release pK_i	[^3H]-RAMH binding pK_i	GPI (NANC) pK_B
PBH	< 5	7.1^a	6.8^c	5.6^d
Betahistidine	4.5	5.2^b	6.2^c	$\leq 4^d$

RAMH = R- α -methylhistamine; PBH = phenylbutanoylhistamine. References: (a) Timmerman (1990); (b) Arrang *et al.* (1985); (c) Kilpatrick & Michel (1991); (d) Taylor & Kilpatrick (1992)

was without antagonist effect and betahistine was very weak, $pK_B = 4.5$. This is of interest to the developing discussion on the existence of H₃ receptor subtypes. PBH and betahistine are relatively potent inhibitors of the H₃ receptor controlling [³H]-histamine release and inhibiting [³H]-RAMH binding (see Table 3), yet are weak in other H₃ receptor-containing preparations such as non-adrenergic non-cholinergic (NANC)-mediated contractions of the guinea-pig isolated ileum. Clearly, the [³H]-acetylcholine release assay fits in with these latter preparations.

Besides inhibiting the effects of RAMH, thioperamide alone enhanced potassium-evoked tritium release indicating that there may be some endogenous histamine tone. This effect was concentration-dependent and the maximum enhancement was approximately 20%. We may be underestimating this effect since the tissue prestimulation before loading with [³H]-choline would deplete endogenous his-

tamine levels. A similar effect of thioperamide may also occur *in vivo* since a recent report reveals that, when administered peripherally to rats, it increases acetylcholine release as measured by microdialysis (Mochizuki *et al.*, 1991).

In conclusion, the selective H₃ receptor agonist, RAMH, inhibited the release of tritium from slices of rat entorhinal cortex preloaded with [³H]-choline. Antagonists for the H₃ receptor, including the selective compound thioperamide, inhibited the effect of RAMH. These antagonist studies also provide evidence for the postulated existence of subtypes of the H₃ receptor, although these observations need to be investigated further before reaching firm conclusions. Thioperamide alone enhanced release in its own right. This latter effect indicates that there may be an inhibitory H₃ receptor tone on acetylcholine release.

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